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The *Asc* locus for resistance to *Alternaria* stem canker in tomato does not encode the enzyme aspartate carbamoyltransferase

Bert Overduin, Saskia A. Hogenhout, Erik A. van der Biezen, Michel A. Haring*, H. John J. Nijkamp, Jacques Hille

Department of Genetics, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

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Abstract. The fungal disease resistance locus *Alternaria* stem canker (*Asc*) in tomato has been suggested to encode the enzyme aspartate carbamoyltransferase (AC Tase). To test this hypothesis a segment of the tomato ACTase gene was amplified by the polymerase chain reaction (PCR) using degenerate primers. The PCR product obtained was subsequently used to isolate an ACTase cDNA clone. Restriction fragment length polymorphism (RFLP) linkage analysis showed that the AC Tase gene and the *Asc* locus do not cosegregate. RFLP mapping positioned the ACTase gene on chromosome 11, while the *Asc* locus is located on chromosome 3. These results exclude the possibility that the ACTase protein is encoded by the *Asc* locus.

Key words: Tomato – *Asc* locus – aspartate carbamoyltransferase – PCR – RFLP linkage analysis

Introduction

Disease resistance in plants has been studied extensively at the genetic and physiological levels. Still virtually nothing is known about the underlying molecular and biochemical processes (for a review see Keen 1992) and so far only one plant resistance gene has been cloned (Johal and Briggs 1992).

The fungal pathogen *Alternaria alternata* f. sp. *lycopersici* causes *Alternaria* stem canker in susceptible cultivars of tomato *(Lycopersicon esculentum)* (Grogan et al. 1975). The disease is characterized by dark brown cankers on stems and necrosis of leaf tissue between the veins. Host-selective AAL toxins that are produced by the fungus play a major role in the pathogenesis (Gilchrist and Grogan 1976). Resistance to the fungus and insensitivity to the toxins is conferred by the *Alternaria* stem canker *(Asc)* locus. While resistance to the fungus is inherited as a single completely dominant gene, insensitivity to the toxins is semi-dominant (Clouse and Gilchrist 1987). The *Asc* locus has been positioned on chromosome 3 (Witsenboer et al. 1989), however, its gene product is still unknown.

For two other host-selective toxins produced by plant pathogens, enzymes involved in nucleotide biosynthesis have been demonstrated as targets. The bacterial toxins tabtoxin, produced by *Pseudomonas tabaci,* and phaseolotoxin, produced by *P. phaseolicola,* were shown to inhibit glutamine synthetase (Turner 1981, 1986) and ornithine carbamoyltransferase (Ferguson and Johnston 1980), respectively. By analogy, the target for the AAL toxins and product of the *Asc* locus has been suggested to be the enzyme aspartate carbamoyltransferase (ACTase) (Gilchrist 1983), a key enzyme in *de novo* pyrimidine biosynthesis. This suggestion was based on three observations: (1) *N*-(phosphonacetyl)-L-aspartate (PALA), a specific inhibitor of ACTase (Collins and Stark 1971), elicits genotype-specific symptoms in tomato leaves similar to those evoked by AAL toxins; (2) intermediates of *de novo* pyrimidine biosynthesis, such as L-aspartate, dihydroorotic acid and orotic acid, reduce the symptoms caused by AAL toxins in leaves (McFarland 1984) and protoplasts (Moussatos 1989); and (3) AAL toxins alter ACTase regulatory kinetics *in vitro* (McFarland 1984). However, experiments on suspension-cultured tomato cells do not support the hypothesis that ACTase is the target site for AAL toxins (Fuson and Pratt 1988).

To investigate the possibility that ACTase is the product of the *Asc* locus, we set out to determine if the gene encoding ACTase and the *Asc* locus cosegregate. In this paper we describe the amplification of a segment of the ACTase gene of tomato by the polymerase chain reaction (PCR) using degenerate primers based on conserved amino acid sequences of the ACTase proteins

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* *Present address*: Section Plant Physiology, Universiteit van Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

Correspondence to: B. Overduin

from other eukaryote organisms, the subsequent isolation of an ACTase cDNA clone using the PCR product obtained as a probe and a restriction fragment length polymorphism (RFLP) linkage analysis of the ACTase gene and the *Asc* locus. Possible relationships between the ACTase gene and the *Asc* locus are discussed.

Materials and methods

Recombinant DNA technology. Recombinant DNA work was performed using standard procedures (Sambrook et al. 1989).

Plant DNA and RNA isolation. Total plant DNA and RNA were isolated as described by Dellaporta et al. (1983) and Kater et al. (1991), respectively, with minor modifications. Poly(A)$^+$ RNA was isolated from total RNA as described by Davis et al. (1986).

PCR amplification. The ACTase gene segment was amplified by PCR from total plant DNA of a resistant *(Asc/Asc) L. esculentum* cultivar (Clouse and Gilchrist 1987). The reaction mixture contained 50 mM TRIS-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 unit Replinase (Dupont), 100 ng of template DNA and 50 pmol of primers ACT1 (5′ GCGAATTC-GC$^{T}/_{A}$GC$^{C}/_{T}$ATG$^{G}/_{CA}/_{CA}/_{T}$CG^{T}/C/$_{G}$TT$^{G}/_{A}$GG^{A}/T/G/$_{C}$GG3′)and ACT5 (5′ CGAAGCTTC$^{G}/_{T}$GAA$^{G}/_{A}$TA$^{A}/_{G}$GC^{A}/G/$_{T}$GC^{T}/C/$_{A}$CG^{T}/G/$_{A}$G/$_{T}$G$^{G}/_{A}$TC 3′) in a reaction volume of 40 µl. The following PCR regime was applied: 20 s 94° C, cooling to 45° C within 1 min; 20 s 45° C, heating to 60° C within 2 min; 5 min 60° C, heating to 94° C; repeated 35 times and followed by 15 min 72° C, using an automatic DNA thermal cycler (Perkin Elmer Cetus). The PCR product was purified from an agarose gel with Prep-A-Gene (Bio-Rad) and amplified for a second time using the same regime. The resulting product was cloned into pUC19.

Southern and Northern blot analysis. For Southern and Northern blot analysis, 10 µg total plant DNA digested with restriction endonucleases (Boehringer) or 4 µg poly(A)$^+$ RNA was electrophoresed through an agarose or formaldehyde gel, respectively, and transferred to Hybond N$^+$ according to the protocol of the manufacturer (Amersham). Hybridization was carried out in 0.5 M $NaHPO_4$ pH 7.2, 7%(w/v) SDS, 1 mM EDTA, 1% (w/v) bovine serum albumin (Church and Gilbert 1984) at 60° C, using a [^{32}P]dCTP (Amersham) radiolabeled ACTase probe prepared by random priming. After hybridization, the filters were washed at stringencies corresponding to either 2 × SSC, 0.1% (w/v) SDS or 0.2 × SSC, 0.1%(w/v) SDS at 60° C and autoradiographed using preflashed Kodak X-Omat AR films at −70° C with intensifying screens.

Screening of a tomato cDNA library. An unamplified *L. esculentum* cDNA library (5×10^4 clones) made from tomato leaf RNA with the UNI-ZAP XR Gigapack 2 cloning kit (Stratagene) was obtained from Dr. Jan van Kan (Department of Phytopathology, Wageningen Agricultural University, The Netherlands) and screened with the radiolabeled PCR product.

DNA sequencing. Sequence analysis was performed by the dideoxy chain termination method (Sanger et al. 1977) using *Taq* polymerase (Boehringer) and fluorescent M13 primers (Promega) employing a DNA Sequenator (Applied Biosystems Model 370A). Database searches were performed with FASTA (Pearson and Lipman 1988).

RFLP linkage analysis. Linkage analysis of the ACTase gene and the *Asc* locus was carried out using F_2 and BC_1 populations derived from a hybrid cross *(L. esculentum × L. pennellii)* segregating for the *Asc* locus (van der Biezen et al., submitted). The F_2 population was made by crossing a susceptible *(asc/asc) L. esculentum* cultivar (Clouse and Gilchrist 1987) with *L. pennellii* (LA716) and subsequent selfing of the F_1. The BC_1 population was obtained by backcrossing the F_1 with the *L. esculentum* parent. Southern blots containing DNA of 20 F_2 and 20 BC_1 plants digested with *Eco*RI, which reveals an RFLP between the *L. esculentum* and *L. pennellii* parents for the ACTase gene, were hybridized with a radiolabeled ACTase probe. Plants were tested for sensitivity to AAL toxins as described by Gilchrist and Grogan (1976).

RFLP mapping. RFLP mapping of the tomato ACTase gene was carried out using an F_2 population of *L. esculentum × L. pennellii* segregating for 64 RFLP markers (Tanksley et al. 1992). Southern blots containing DNA of 32 F_2 plants digested with *Eco*RV, which shows an RFLP between the *L. esculentum* and *L. pennellii* parents for the ACTase gene, were hybridized with a radiolabeled ACTase probe. The segregation data were translated into the map position of the ACTase gene using the interactive computer package MAPMAKER (Lander et al. 1987). Plants and computer program were kindly provided by Prof. Steven Tanksley (Department of Plant Breeding and Biometry, Cornell University, Ithaca, N.Y., USA).

Results

Amplification of a tomato ACTase gene segment using degenerate primers

To amplify a segment of the ACTase gene from tomato, primers were designed utilizing conserved amino acid sequences of the ACTase proteins from a number of eukaryote organisms: *Dictyostelium discoideum* (Faure et al. 1989), *Drosophila melanogaster* (Freund and Jarry 1987), *Saccharomyces cerevisiae* (Nagy et al. 1989) and Syrian hamster (Simmer et al. 1989) (Fig. 1). Two regions of eight amino acids were selected which were highly conserved (88%) between the different organisms. Two primer pools, designated ACT1 and ACT5, were designed that contained nearly all possible nucleotide sequences encoding these two regions (degeneracy n = 752 and 864

D. discoideum NH2-65aa-A-A-M-Q-R-L-G-G-208aa-D-P-R-A-A-Y-F-R-21aa-COOH

D. melanogaster NH2-63aa-A-A-M-L-R-L-G-G-206aa-D-P-R-A-A-Y-F-R-34aa-COOH

S. cerevisiae NH2-63aa-A-A-M-E-R-L-G-G-209aa-D-H-R-A-A-Y-F-R-21aa-COOH

Syrian hamster NH2-64aa-A-A-M-A-R-L-G-G-208aa-D-P-R-A-A-Y-F-R-20aa-COOH

5'-GCGAATTC.GCA.GCC.ATG.CAA.CGC.TTA.GGA.GG-3'
*Eco*RI T T GCT G G C T G T

3'-CTA.GGA.GCA.CGA.CGA.ATA.AAG.GCT.TCGAAGC-5'
G TG C G G G T *Hin*dIII T T T

ACT1 (n=768)

ACT5 (n=864)

Fig. 1. Nucleotide sequence of the PCR primer pools ACT1 and ACT5. Degenerate PCR primer pools ACT1 and ACT5 were designed based on highly conserved sequences of aspartate carbamoyltransferases (ACTases) from four different eukaryote organisms, *Dictyostelium discoideum, Drosophila melanogaster, Saccharomyces cerevisiae* and Syrian hamster. In the upper part, the sequences of these conserved regions are given. In addition the distances from regions to the N-terminal and C-terminal ends are given. It should be noted that in all four cases ACTase is part of a larger, multifunctional protein, called CAD, that carries the first two or three enzymatic activities of the *de novo* pyrimidine biosynthetic pathway (carbamoylphosphate synthetase, aspartate carbamoyltransferase and dihydroorotase). In the lower part the sequences of the primer pools ACT1 and ACT5 are presented

```
    G Y L M A T L F Y E P S T R T R L S F E
  1 GGTTATCTTATGGCTACTCTGTTTTATGAACCTTCAACTAGAACTAGGCTTTCATTTGAA
    S S M K R L G G E V L T T E N A R E F S
 61 TCTTCTATGAAGCGTTTAGGAGGAGAAGTACTAACAACTGAAAATGCTCGTGAATTTTCG
    S A A K G E T L E D T I R T V E G Y S D
121 TCTGCGGCGAAAGGTGAAACACTAGAAGATACAATTAGAACTGTTGAAGGTTACTCTGAT
    I I V M R H F E S G A A R R A A L T A S
181 ATCATTGTTATGAGGCATTTCGAAAGTGGTGCTGCTCGACGAGCTGCATTGACTGCATCT
    I P I I N A G D G P G Q H P T Q A L L D
241 ATTCCGATTATAAATGCAGGAGATGGTCCGGGACAACACCCGACTCAGGCTCTTCTAGAT
    V Y T I G R E I G K L D G I N I A L V G
301 GTGTATACGATTGGACGAGAAATAGGGAAACTCGATGGTATAAACATTGCTCTTGTTGGT
    D L A Y G R T V R S L A H L L A L Y K D
361 GATTTAGCATACGGGAGGACAGTTCGTTCACTTGCTCATTTGCTTGCGCTGTATAAAGAT
    V K I Y F V S P D V V K M K D D I K D Y
421 GTGAAGATTTACTTTGTATCCCCTGATGTTGTTAAAATGAAGGATGACATAAAGGATTAC
    L T S M G V R W E E S A D L I E V A S K
481 TTGACATCAATGGGGGTTCGATGGGAGGAAAGTGCTGATTTGATCGAGGTGGCTTCTAAA
    C D V V Y Q T R I Q R E R F G E R V D L
541 TGTGACGTGGTGTATCAAACTCGGATTCAACGAGAGAGATTTGGAGAGAGGGTTGATTTG
    Y E E A R G K Y I V D M S V V N A M Q K
601 TATGAAGAAGCTCGAGGTAAGTATATCGTTGATATGAGTGTCGTAAATGCTATGCAGAAA
    H A V V M H P L P R L D E I T V D V D G
661 CATGCTGTAGTGATGCATCCTTTGCCAAGACTTGATGAGATAACGGTTGATGTCGATGGT
    D P R A A Y F R Q A K N G L Y I R M A L
721 GATCCGAGGGCTGCTTATTTCAGACAAGCTAAGAATGGTCTCTACATTCGGATGGCGCTT
    L K L L L L G W *
781 TTGAAGCTTCTACTCCTTGGTTGGTGAAAGACGCGAACTTTTTATGTGGCGAAATCGTGC
841 TGCTTGTTGTCTGGATTCTGCTGTTGTTGAATTCTAGATTTAGTTAAACTTGTTTTAGCA
901 TAAAAGAAAATTGAATCTCTTTCTTTGTTCACAAAATAGTACATTCTATATATGCAATTT
961 GAAAACTTTGAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequence of the tomato ACTase cDNA clones pAT6 and pAT7. The sequences annealing to the PCR primers ACT1 and ACT5 are *underlined.* The amino acids forming the active site of the ACTase enzyme are enclosed by *circles*

for ACT1 and ACT5, respectively). To facilitate the cloning of PCR products, *Eco*RI and *Hin*dIII restriction enzyme sites were added to the 5′ ends of ACT1 and ACT5, respectively.

PCR on tomato genomic DNA resulted in a product of 0.7 kb, that was subsequently cloned and sequenced. The nucleotide sequence obtained was translated into an amino acid sequence and compared with the amino acid sequence of the ACTase proteins of the other organisms. The high degree of homology, especially in the conserved areas (data not shown), strongly suggested that a segment of the tomato ACTase gene had been amplified.

Isolation of a tomato ACTase cDNA clone

Hybridization of Northern blots with the PCR product showed equal amounts of transcript of one size in RNA isolated from tomato leaves, stems and roots (data not shown). To isolate an actively transcribed ACTase gene a tomato leaf cDNA library was screened using the PCR product as a probe. Screening of approximately 50000 clones resulted in 2 positive clones, designated pAT6 and pAT7. Restriction analysis and DNA sequencing showed that both cDNAs were identical, 988 bp in size, and shared, between the primer annealing sites, 100% homol-

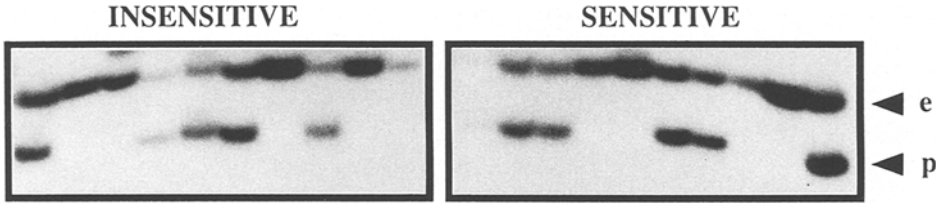

Fig. 3. Restriction fragment length polymorphism (RFLP) linkage analysis of the tomato ACTase gene and the *Asc* locus. A Southern blot containing *Eco*RI-digested DNA from 20 plants derived from a BC_1 population of *Lycopersicon esculentum* × *L. pennellii*, segregating for the *Asc* locus, was hybridized with a radiolabeled ACTase probe. RFLPs for *L. esculentum* and *L. pennellii* are indicated by e and p, respectively. Sensitivity to AAL toxins was determined by a leaf bioassay

ogy with the PCR product. The presence of a poly(A) tail indicated that the cDNAs were complete at the 3′ end. However, the absence of a start codon indicated that a part of the 5′ end was lacking (Fig. 2). Comparison with DNA sequences in the EMBL and GenBank databases showed a high percentage homology (on average 56%) with the ACTase genes of both eukaryote and prokaryote organisms. Moreover, from the deduced amino acid sequence it appeared that all 14 amino acids which are reported to form the active site of the ACTase enzyme of *Escherichia coli* and *D. discoideum* (Faure et al. 1989) were 100% conserved in the amino acid sequence deduced from the isolated cDNAs, so it could be concluded that indeed the tomato ACTase gene had been cloned. From the fact that the size of the PCR product, made with genomic tomato DNA as a template, was the same as the distance between the primer annealing sites in the cDNA clone (699 bp) it can be concluded that the ACTase gene of tomato contains no introns in the region between these annealing sites.

Copy number determination of the tomato ACTase gene

For RFLP linkage analysis and mapping purposes a copy number determination was performed to ensure that only one copy of the ACTase gene is present in the tomato genome. To this end the radiolabeled ACTase cDNA was used as a probe on a Southern blot containing tomato genomic DNA digested with three different restriction enzymes, *Bam*HI, *Hae*III, and *Pst*I. As none of these enzymes cuts inside ACTase cDNA, separate copies should show up as different bands. From the fact that in all three cases, after low stringency (2 × SSC, 0.1% SDS) as well as high stringency washes (0.2 × SSC, 0.1% SDS), only one distinct band could be seen (data not shown), it was concluded that only one copy of the ACTase gene is present in the tomato genome.

RFLP linkage analysis

To determine whether the ACTase gene and the *Asc* locus are one and the same locus, an RFLP linkage analysis was performed. Populations of 20 F_2 and 20 BC_1 plants derived from *L. esculentum* × *L. pennellii*, were used. Because a susceptible *(asc/asc) L. esculentum* cultivar was used and *L. pennellii* is resistant *(Asc/Asc)*, both the F_2 population, obtained by selfing the F_1, and the BC_1 population, obtained by backcrossing the F_1 with the *L. esculentum* parent, segregated for the *Asc* locus. Leaf bioassays showed that half of both the F_2 and BC_1 plants were sensitive and half were insensitive to AAL toxins, as expected. Subsequently, Southern blots containing DNA of these plants were hybridized with a radiolabeled ACTase probe. As in both the F_2 (data not shown) and the BC_1 populations (Fig. 3) no cosegregation of the ACTase gene and insensitivity to AAL toxins was observed, it can be concluded that the ACTase gene and the *Asc* locus are different unlinked loci.

Localization of the tomato ACTase gene

The genomic position of the ACTase gene was determined by RFLP mapping. To this end, Southern blots containing DNA of an F_2 population derived from *L. esculentum* × *L. pennellii* were hybridized with a radiolabeled ACTase probe. It appeared that the ACTase gene is located on chromosome 11, 22 cM distal to RFLP marker TG194 (Tanksley et al. 1992), confirming the conclusion that the ACTase gene and the *Asc* locus are different loci.

Discussion

In this paper we show that the enzyme ACTase cannot be the product of the *Asc* locus in tomato. This was demonstrated by performing an RFLP linkage analysis, in which no cosegregation of the ACTase gene and the *Asc* locus was observed. Additional evidence is provided by the observation that the ACTase gene is located on chromosome 11 of tomato, whereas the *Asc* locus has been positioned on chromosome 3.

Our results show that it is possible to isolate (segments of) plant genes by performing PCR on total plant DNA using primers deduced from highly conserved amino acid areas in proteins from other organisms. Despite the high degeneracy of the primer pools (n = 752 and n = 864), a specific PCR product was obtained without the need to reduce the complexity of the primer pools by incorporation of deoxyinosine in codons with three or four base ambiguities, as was reported by Aarts et al. (1991).

Northern blot analysis showed that the ACTase gene is transcribed in tomato leaves, stems and roots, as can

be expected for a housekeeping gene. From Southern blot analysis it appeared that the tomato genome contains only a single copy of the ACTase gene.

Although we have shown that the ACTase protein is not the product of the *Asc* locus in tomato, the possibility that ACTase is the target site of AAL toxins still remains open. Presently, two enzymes involved in nucleotide biosynthesis are known to be targets of toxins produced by plant pathogens. Glutamine synthetase and ornithine carbamoyltransferase are inhibited by tabtoxin, produced by *P. tabaci* (Turner 1981, 1986) and phaseolotoxin, produced by *P. phaseolicola* (Ferguson and Johnston 1980), respectively.

With respect to the nature of the product of the *Asc* locus, we can only speculate. So far the protein product of only one plant disease resistance gene has been identified. In maize, resistance to the fungus *Cochliobolus carbonum* race 1, which causes leaf spot and ear mold, is conferred by the *HM1* gene. Recently, the *HM1* gene was cloned and shown to encode NADPH-dependent HC toxin reductase (Johal and Briggs 1992), which inactivates HC toxin, a cyclic tetrapeptide produced by the fungus to permit infection. By analogy, the *Asc* locus might encode a compound capable of detoxifying AAL toxins by either destruction or chemical modification. This would imply that resistant plants synthesize an active gene product while susceptible plants lack such a product. However, the reverse might also be possible. Susceptible plants might make an active gene product which activates AAL toxins by chemical modification. Other possible resistance mechanisms include the presence or absence of a toxin target site, the ability or inability to transport the toxin to the target site, insensitivity of a target site, overproduction of a target site and the capacity or incapacity for metabolic recovery from an initial biochemical lesion (Daly 1984).

In order to isolate the *Asc* locus, experiments are in progress in our laboratory to inactivate either its dominant or recessive allele by transposon tagging using the maize transposable elements *Ac* and *Ds* (Haring et al. 1991).

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